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Mercury Adaptation among Bacteria from a Deep-Sea Hydrothermal Vent

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Since deep-sea hydrothermal vent fluids are enriched with toxic metals, it was hypothesized that (i) the biota in the vicinity of a vent is adapted to life in the presence of toxic metals and (ii) metal toxicity is modulated by the steep physical-chemical gradients that occur when anoxic, hot fluids are mixed with cold oxygenated seawater. We collected bacterial biomass at different distances from a diffuse flow vent at 9°N on the East Pacific Rise and tested these hypotheses by examining the effect of mercuric mercury [Hg(II)] on vent bacteria. Four of six moderate thermophiles, most of which were vent isolates belonging to the genus *Alcanivorax*, and six of eight mesophiles from the vent plume were resistant to >10 μM Hg(II) and reduced it to elemental mercury [Hg(0)]. However, four psychrophiles that were isolated from a nearby inactive sulfide structure were Hg(II) sensitive. A neighbor-joining tree constructed from the deduced amino acids of a PCR-amplified fragment of *merA*, the gene encoding the mercuric reductase (MR), showed that sequences obtained from the vent moderate thermophiles formed a unique cluster (bootstrap value, 100) in the MR phylogenetic tree, which expanded the known diversity of this locus. The temperature optimum for Hg(II) reduction by resting cells and MR activity in crude cell extracts of a vent moderate thermophile corresponded to its optimal growth temperature, 45°C. However, the optimal temperature for activity of the MR encoded by transposon Tn*501* was found to be 55 to 65°C, suggesting that, in spite of its original isolation from a mesophile, this MR is a thermophilic enzyme that may represent a relic of early evolution in high-temperature environments. Results showing that there is enrichment of Hg(II) resistance among vent bacteria suggest that these bacteria have an ecological role in mercury detoxification in the vent environment and, together with the thermophilicity of MR, point to geothermal environments as a likely niche for the evolution of bacterial mercury resistance.

Heavy metals are highly enriched in hydrothermal vent fluids of mid-oceanic ridge systems (35), reaching concentrations that are considered to be toxic to living organisms (26). The steep physical-chemical gradients that occur when reduced, hot, element- and sulfur-rich vent fluids are diluted with oxygenated, cold seawater create a gradient in metal toxicity in the vent environment (20). As oxygen mixes with the anoxic, sulfur-rich fluid, metal speciation can shift from metal sulfides that show poor bioavailability and low toxicity to more soluble and oxidized forms with increased bioavailability and toxicity. With such a change in metal speciation, high tolerance to metals is expected among microbes inhabiting niches of the vent ecosystem where mixing between hydrothermal fluids and oxygenated seawater occurs, such as diffuse flow vents and associated plumes. This hypothesis is supported by experimental data showing that thermophilic archaea and bacteria from highly reduced vent microhabitats were metal susceptible (12, 19), whereas high levels of metal resistance were found for the ectosymbiotic bacteria of the polycheate annelids *Alvinella pompejana* and *Alvinella caudata* (16) and for free-living, mesophilic bacteria (28) and flagellated protists (3). Here we sought further evidence that supports this hypothesis by examining the mercury resistance of bacteria that were isolated at various proximities to diffuse flow vents at 9°50′N on the East Pacific Rise (EPR).

Mercury in anoxic hydrothermal fluids originates in cinnabar deposits in the underlying rocks of the oceanic subsurface, where it is present as sulfidic complexes, dissolved ionic, and vapor monoatomic elemental mercury (6). The total concentrations of mercury in vent fluids at 13°N on the EPR were reported to range from 7.2 to 148.4 ng liter^{-1} (8); these concentrations were up to 1,000 times higher than the concentrations in ambient seawater (18) and were comparable to the concentrations observed in highly contaminated surface waters (30). The seafloor in the proximity of hydrothermal vents is enriched with mercury (32), and a sample collected from a polymetallic sulfide structure at 9°50′ N on the EPR contained 53.5 ppm of total Hg (N. Bloom, personal communication). A high input of mercury was also evident based on the accumulation of mercury in vestimeniferan worm tissues at concentrations that were more than 10^5-fold higher than the concentrations in seawater (2).

Microorganisms transform mercury among its three oxidation states, 0, +1, and + 2, and between inorganic and organic mercury forms (4), thereby influencing the toxicity of mercury

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and its environmental mobility. The broadly distributed bacterial mercury resistance (*mer*) operon encodes a flavoenzyme, mercuric reductase (MR), that reduces ionic mercury [Hg(II)] to the elemental, less toxic form [Hg(0)], which then partitions into the vapor phase at the solid-gas and liquid-gas interfaces (6). In highly contaminated surficial environments, bacteria that possess (17) and express (30) the MR gene, *merA*, are enriched, and their activities enhance the removal of mercury, thereby decreasing its burden to the ecosystem (4). In the present study we found high levels of *merA*-specified resistance to Hg(II) in bacteria isolated from the interface between hydrothermal fluids and oxygenated seawater, and in this paper we report that MR from a mesophilic bacterium is a thermophilic enzyme. Together, these results suggest that the broadly distributed *mer* system may have evolved in geothermal environments.

MATERIALS AND METHODS

Collection of samples. Samples of hydrothermal fluids from diffuse flow vents (vent samples), of water collected from vent-associated plumes at about 1 m above the vent (plume samples), and of seawater proximal to inactive sulfide vent structures (control samples) were collected from the EPR (9°50′N, 104°17′W) at a depth of 2,500 m during a cruise aboard R/V *Atlantis* in May 1999. The samples were collected by using either titanium samplers (hydrothermal fluids) or Niskin samplers (plumes and bottom seawater samples) operated by the manipulator of the DSV *Alvin*. On the surface, samples were promptly transferred in the ship's laboratory, and subsamples were placed in tubes closed with stoppers and stored at 4°C.

Isolation and characterization of pure cultures. An aliquot (0.1 ml) of each sample was used to inoculate liquid and solid artificial seawater medium (ASW), which contained (per liter) 24.0 g of NaCl, 0.7 g of KCl, 7.0 g of $MgCl_2$, 3.0 g of yeast extract, and 2.5 g of peptone. Inoculated plates and tubes were incubated at 4, 10, 28, 37, and 45°C, and pure cultures were obtained by repeated transfers of single colonies onto fresh media. The optimal temperature for growth (T_{opt}) was determined for each isolate by monitoring the growth at each of the temperatures indicated above. The 16S rRNA gene was selectively amplified from the genomic DNA of each isolate by PCR as described previously (34) by using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1517R (5′-ACGGC TACCTTGTTACGACTT-3′). The sequence of the amplified 16S rRNA gene was determined for both strands with an ABI 3100 Avant genetic analyzer (Applied Biosystems, Foster City, Calif.). With the exception of four strains, the full sequence of the 1.5- kb 16S rRNA gene amplification product was used for determination of the genus of the closest relative by Blastn analysis. The remaining four strains were related to known genera by using 800- to 1,000-bp sequences of the 16S rRNA gene.

Reference strain and plasmid. *Escherichia coli* strain JM109 carrying plasmid pKSM::Tn*501*was grown in Luria-Bertani medium at 37°C. The *mer* transposon Tn*501* was originally discovered in plasmid pVS1 carried by *Pseudomonas aeruginosa* strain PAT (31).

Determination of mercury resistance and volatilization. Ten-microliter drops of 1:100 dilutions of mid-log-phase cultures in ASW were placed on solid ASW containing the following $HgCl_2$ concentrations: 0, 0.5, 1, 2, 5, 10, 20, 30, 40, 50, and 75 μM. The plates were incubated at the optimal growth temperature for each strain tested until confluent growth was observed in control (no $HgCl_2$) plates, and then growth on the $HgCl_2$-supplemented plates was examined. To determine how much Hg(II) was lost abiotically from ASW incubated at 45°C, sterile agar plugs containing 50 μM $^{203}HgCl_2$ (specific activity, 0.12 nCi μmol^{-1}; Isotope Products, Valencia, Calif.) were incubated at 45°C for 3 days. The plugs were dissolved in EcoLume scintillation liquid (ICN Pharmaceuticals) and counted with a Beckman LS6500 multipurpose scintillation counter (Beckman Instruments Inc., Fullerton, Calif.). The qualitative ability of all strains to reduce Hg(II) to Hg(0) was determined by the darkening of X-ray film when Hg(0) produced by resting cell suspensions formed amalgam with the silver emulsion in the film, as described by Nakamura and Nakahara (25).

To quantitatively monitor the loss of Hg during growth, cultures that were grown overnight in 5 ml of ASW with 25 μM $HgCl_2$ were spiked with an additional 25 μM $HgCl_2$, incubated for an additional 30 min, and diluted 1:100 into fresh ASW containing 25 μM $^{203}HgCl_2$ (specific activity, 90 nCi μmol^{-1}). These cultures were then grown at the optimal growth temperature for 8 to 10 h. Each hour, the growth and the remaining $^{203}HgCl_2$ were determined by recording the optical density at 660 nm (A_{660}) and by scintillation counting of 1-ml aliquots of the growing culture, respectively.

Detection and sequencing of *merA* genes. Two primer sets were used to amplify both short (288-bp) and long (1,200-bp) *merA* PCR products. The reverse primer A5-n.R (5′-ACCATCGTCAGRTARGGRAAVA-3′) was used in both PCRs. The short product was obtained by using primer A1s.F (5′-TCCGCAAGTNGC VACBGTNGG-3′) and the following reaction conditions: 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s for 25 cycles. The long product was obtained by using the forward primer A2-n.F (5′-CCATCGGCGGCWSYTGCGTSAA-3′) and the same reaction conditions that were used for the short product, except that the extension time at 72°C was 1.5 min. The degenerate primers were designed to encompass the known diversity of *merA* in gram-negative bacteria (J. K. Schaefer, unpublished data). Genomic DNA from each isolate was used as a template in all PCRs, and amplification products of the expected size were cloned by using a TA cloning kit as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, Calif.) and sequenced.

Phylogenetic analysis. A neighbor-joining MerA tree was constructed from the deduced amino acid sequences (length, 93 to 95 residues) of the 288-bp *merA* amplification products of vent strains and from reference MerA sequences from GenBank (http://www.ncbi.nih.gov/). Reference sequences were selected to represent the major clusters in the MerA phylogenetic tree. The database sequences included in the tree were the sequences of *Aeropyrum pernix* (accession no. NP_147957), *Sulfolobus sulfataricus* (AE006863), *Staphylococcus aureus* plasmid pI258 (PO8663), *mer* transposon Tn*5042* (AJ563381), *Pseudomonas* sp. strain ED23-33 (CAC14700), *Acidothiobacillus ferrooxidans* (D90110), *Pseudomonas putida* MU10-2 (AJ318529), *mer* transposons Tn*21* (KO3089) and Tn*501* (Z00027), *Bacillus cereus* RC607 (AF138877), and *Pseudomonas haloplanktis* M1 (AY005468). The sequences were aligned with ClustalX (33) by using default program settings. A bootstrapped MerA tree was constructed by using the distance function of PAUP* (version 4.0 beta 10; Sinaur Associates, Sunderland, Mass.).

Mercuric reductase: resting cell activities. Assays with marine strains were performed by using the protocol of Weiss et al. (36), with modifications. The modifications included replacing sodium phosphate in the assay buffer with 50 mM HEPES (Sigma) (pH 7.4) and including 0.1 N NaCl to increase the osmolarity of the assay solution. Resting cell assays with *E. coli* JM109/pKSM::Tn*501* were carried out as described previously (36). Cultures were grown and induced at their optimal growth temperatures for these assays. $^{203}HgCl_2$ (10 μM; specific activity, 0.7 nCi μmol^{-1}) was added to suspensions of washed resting cells (A_{660}, 0.1) that were incubated at 20, 28, 37, 45, and 50°C with vigorous shaking. The remaining $^{203}HgCl_2$ was monitored in aliquots removed every 5 min following initiation of the assay. Specific volatilization rates were calculated from the linear range of curves describing $^{203}HgCl_2$ loss over time.

Mercuric reductase: crude cell extracts. Overnight cultures were diluted 1:20 into fresh ASW (strain EPR7) or Luria-Bertani medium (strain JM109/pKSM:: Tn*501*) containing 10 μM $HgCl_2$ and were grown to an A_{660} of 0.4 at the optimal growth temperatures with vigorous shaking. $HgCl_2$ was added to a final concentration of 10 μM, and the cells were incubated for an additional 10 min. Induced cell suspensions were placed on ice, and the chilled cells were centrifuged in preweighed tubes; the pellets were washed once with phosphate-buffered saline, weighed, and stored at −20°C until analysis. The pellets were resuspended to a concentration of approximately 200 mg (wet weight) ml^{-1} in buffer consisting of 20 mM sodium phosphate (pH 7.5), 0.5 mM EDTA, and 1 mM β-mercaptoethanol, and the cells were broken by intermittent sonication (Vibra Cell; Sonics & Materials Inc., Danbury, Conn.) for a total of 3 min on ice. The sonicated cell suspensions were centrifuged with an Eppendorf centrifuge (14,000 rpm for 30 min at 4°C), and each supernatant was removed and placed on ice. Assays were performed as described by Fox and Walsh (14) in a solution containing 80 mM sodium phosphate (pH 7.4), 1 mM β-mercaptoethanol, 200 μM NADPH, and 100 μM $HgCl_2$; the $HgCl_2$-dependent oxidation of NADPH was monitored by determining the decrease in A_{340} with a UV-visible spectrophotometer (UV-265; Shimadzu, Columbia, Md.). Specific activities were expressed in units per 100 μg of protein, where 1 U of activity was defined as 1 μmol of NADPH oxidized min^{-1}. Final concentrations of 173 to 338 and 38 to 144 μg of extract protein ml^{-1} were used to assay the MR of Tn*501* and EPR7, respectively. For each assay condition, the rate of NADPH oxidation was determined with and without $HgCl_2$, and reductase activities were calculated by subtracting the rate observed without Hg from the rate observed with Hg. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories Inc., Hercules, Calif.).

To measure activities at various temperatures, concentrated cell extracts were incubated at the assay temperature for 10 min, after which 5 to 20 μl of each extract was added to 800 μl of reaction buffer that was separately incubated at the desired temperature. The spectrophotometer was equipped with a temper-

TABLE 1. General characteristics and the responses to $HgCl_2$ of psychrophilic, mesophilic, and moderately thermophilic bacterial isolates from 9°50′N on the East Pacific Rise

Optimum growth temp (°C)	Isolate	Sampling site	Taxon of closest relative (% sequence similarity)[a]	MIC [μM Hg(II)][b]	Hg(II) volatilization[c]
4	760C	Control	*Moritella* (99)	2	ND
4	760D	Control	*Psychrobacter* (97)	2	ND
4	761F	Control	*Photobacterium* (99)	2	ND
4	762G	Control	*Moritella* (99)	2	ND
28	763D	Control	*Psychrobacter* (99)	5	+/−
28	EPR13	Plume	Unclassified *Rhizobiales* (98)	5	+
28	EPR11	Vent	*Halomonas* (99)	40	++
28	EPR1	Plume	*Pseudoalteromonas* (99)	50	++
28	EPR2	Plume	*Pseudoalteromonas* (97)	50	++
28	EPR3	Plume	*Pseudoalteromonas* (97)	50	++
28	EPR15	Plume	*Marinobacter* (99)	50	++
28	EPR12	Plume	*Pseudomonas* (98)	75	++
45	EPR9	Vent	*Bacillus* (99)	5	+
45	EPR5	Plume	*Alcanivorax* (99)	10	+/−
45	EPR6	Vent	*Alcanivorax* (99)	75	++
45	EPR7	Vent	*Alcanivorax* (99)	75	++
45	EPR8	Vent	*Alcanivorax* (99)	75	++
45	EPR10	Plume	*Alcanivorax* (99)	75	++

[a] Based on 16S rRNA sequence similarity.
[b] The values are the lowest concentrations of Hg(II) at which growth on ASW plates was inhibited.
[c] The data indicate the intensity of dark spots created on X-ray film as a result of exposure to Hg(0) formed by a cell suspension that was incubated with 250 μM $HgCl_2$. +/−, weak signals; +, clear signals; ++, very intense signals; ND, not determined.

ature-controlled cuvette holder with a circulating water bath and a water jacket. Following incubation at ≥55°C, a precipitate that formed in crude cell extracts of strain JM109/pKSM::Tn*501* was removed by a brief centrifugation at room temperature. Assays were then performed with the cleared supernatant. The concentration of proteins in the unheated extract was used in calculating specific activities at all temperatures.

Preparation and assay of Tn*501* MerA catalytic core. The catalytic core of Tn*501* MerA is comprised of an initiating Met followed by residues E96 to G561 of the full-length Tn*501* enzyme (21). The subcloned gene was incorporated into a pET-11d vector and was expressed in the BL21(DE3)/pLys strain of *E. coli* by using a standard isopropyl-β-D-1-thiogalactopyranoside induction protocol. The protein was purified essentially as previously described for the full-length protein (22), except that an Orange 3 resin (made by Prometic and sold by Sigma) replaced the discontinued Orange A Dye Matrex resin. The enzyme concentration was calculated as the concentration of active sites by using $\varepsilon_{456} = 11.3\ mM^{-1}\ cm^{-1}$ for the enzyme-bound flavin adenine dinucleotide. For the assays, a concentrated enzyme stock (10.3 μM) was made in 80 mM potassium phosphate buffer (pH 7.3) containing 0.1% bovine serum albumin. At each temperature tested, an aliquot of the concentrated enzyme was heated in an Eppendorf tube placed in a water bath, while 0.985 ml of the reaction mixture containing 80 mM potassium phosphate (pH 7.3), 0.1% bovine serum albumin, 100 μM NADPH, and 1 mM cysteine was heated at the appropriate temperature in a temperature-controlled cuvette in a Shimadzu UV-2101PC spectrophotometer. After 2 min of preheating, 5 μl of the enzyme was added to the reaction mixture to give a final enzyme concentration of 51.5 nM (active sites), and the absorbance at 340 nm was monitored for background NADPH oxidase activity for 1 min. Hg(II) reductase activity was initiated by addition of 10 μl of 10 mM $HgCl_2$ to give a final Hg(II) concentration of 100 μM and was measured from the NADPH consumption monitored by the decrease in A_{340} ($\Delta\varepsilon_{340} = 6.2\ mM^{-1}\ cm^{-1}$). The Hg(II) reductase specific activity was defined as the difference between the rates before and after addition of $HgCl_2$ divided by the concentration of enzyme active sites. A standard 2-min preheating time was used as this time was determined to be the maximum length of incubation at 65°C with no loss of activity.

Nucleotide sequence accession numbers. Sequences of the 16S rRNA gene have been deposited in the GenBank database under the following accession numbers: strain 760C, AY394859; strain 760D, AY700222; strain 761F, AY700223; strain 762G, AY394860; strain 763D, AY394861; strains EPR1 through EPR3, AY394862 to AY394864; strain EPR5, AY700224; strain EPR6, AY394865; strain EPR7, AY394866; strain EPR8, AY700225; and strains EPR9 through EPR15, AY394867 to AY394873. The *merA* sequences of strains EPR3, EPR6, EPR7, and EPR8 have been deposited in the GenBank database under accession numbers AY700226 through AY700229.

RESULTS

Mercury resistance in deep-sea vent bacteria. Several aerobic, heterotrophic microorganisms with T_{opt} in the range from 4 to 45°C were obtained in pure culture under nonselective conditions (Table 1). Most of the moderately thermophilic strains (T_{opt}, 45°C) were related to the genus *Alcanivorax*, while the mesophilic strains ($T_{opt.}$, 28°C) were related to the genera *Pseudoalteromonas*, *Halomonas*, *Pseudomonas*, *Marinobacter*, and unclassified *Rhizobiales*. Strictly psychrophilic strains ($T_{opt.}$, 4°C), related to the genera *Moritella*, *Psychrobacter*, and *Photobacterium*, were isolated in the vicinity of cold, inactive sulfide structures. A clear relationship among spatial distribution, T_{opt}, and tolerance to Hg(II) of the test strains emerged. Most bacteria isolated from the source fluids (vent) were moderately thermophilic and highly resistant to Hg(II), most bacteria isolated from the plume were mesophilic with lower levels of tolerance to Hg(II), and the four control strains were psychrophilic (T_{opt}, 4°C) and sensitive to Hg(II). All strains that were resistant to ≥40 μM $HgCl_2$, as well as some strains with a moderate level of resistance (5 μM $HgCl_2$), reduced Hg(II) to Hg(0) (Table 1).

Mercury volatilization. One mesophile, strain EPR3 (Fig. 1A), and three moderately thermophilic strains, EPR6, EPR7, and EPR8 (Fig. 1B), were examined for the ability to remove Hg(II) during growth. For EPR3, an initial rapid loss of Hg(II) preceded growth, a pattern often documented for Hg(II)-resistant bacteria (4). Loss of Hg(II) preceding growth was also observed with the moderately thermophilic strains (data not shown). A substantial amount of Hg(II) was lost from the uninoculated ASW, and this loss increased as the temperature increased from 28 to 45°C. Loss from ASW agar plugs was also observed; 34% of the added Hg(II) was lost during 3 days of incubation at 45°C. Various chemical transformations that are stimulated in the presence of organic matter may account for

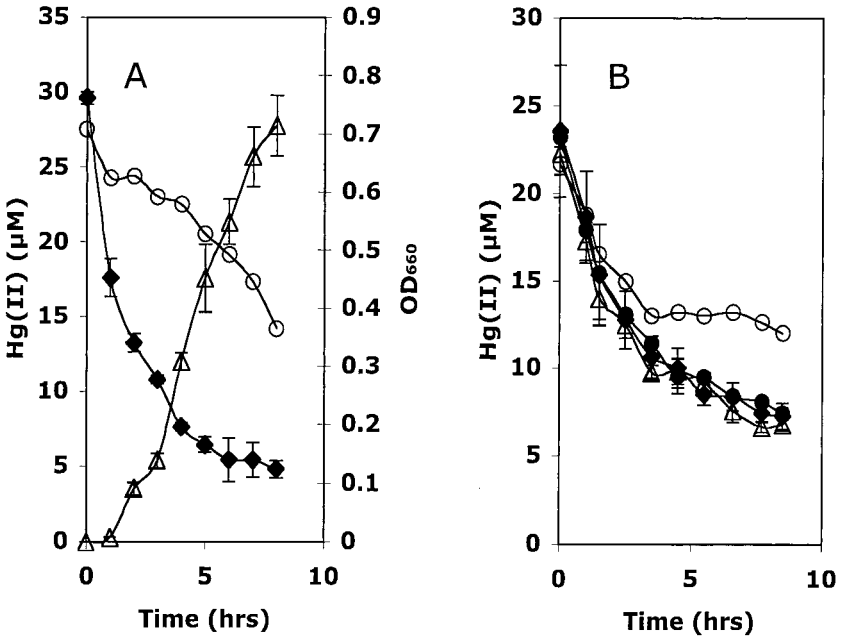

FIG. 1. Loss of Hg(II) during growth of vent bacteria. (A) Mesophilic strain EPR3. Symbols: ◆, Hg(II) remaining in solution; △, OD_{600} (optical density at 600 nm). (B) Remaining Hg(II) in growing cultures of moderately thermophilic strains EPR6 (◆), EPR7 (△), and EPR8 (●). ○, remaining Hg(II) in uninoculated medium. Means and standard deviations for three replicate samples are shown.

nonbiological loss of Hg(II) from ASW at elevated temperatures (4). Nevertheless, significantly more Hg(II) was lost in the presence of active cultures of the Hg(II)-resistant strains (Fig. 1), supporting the notion that *mer*-mediated resistance to Hg(II) and concomitant reduction of Hg(II) occur in bacteria from deep-sea hydrothermal vents.

Genes encoding mercuric reductase in deep-sea strains. A 288-bp PCR fragment of the *merA* gene was obtained from three moderately thermophilic strains that were related to the genus *Alcanivorax* and from one mesophile, strain EPR3, which was related to the genus *Pseudoalteromonas* (Table 1). Repeated attempts to amplify *merA* from the remaining resistant strains failed, suggesting that there is divergence of MerA sequences in these strains beyond that which is presently available in databases. Phylogenetic analysis of the deduced amino acid sequences (Fig. 2) revealed that the genes from the moderate thermophiles (strains EPR6, EPR7, and EPR8) formed a unique cluster that was most closely related to, yet distinct from, the clade that includes the best-characterized MR (17). While closely related, the MerA fragments of these *Alcanivorax* strains differed from each other by one to three amino acid residues. The sequence of the mesophilic strain EPR3 clustered with the MR sequence of the marine bacterium *P. haloplanktis* M1 (15). In addition, a long *merA* amplification product (1,200 bp) was amplified from strain EPR15, and its 5′ terminus was sequenced and was found to be 100% identical to the corresponding region of *merA* from *P. haloplanktis* M1 (data not shown). The 3′ terminus of the gene from EPR15, encompassing the 288-bp region homologous to the short *merA* amplification product, was not sequenced, and therefore it was not included in the tree in Fig. 2. No *merA* gene was amplified by PCR from any of the Hg(II)-sensitive psychrophilic control strains.

Temperature effects on mercuric reductase activities. Since the optimal catalytic activities of enzymes from thermophilic microorganisms often occur near the optimal growth temperature (11), the temperature sensitivities of the MR enzymes of the mesophilic strain EPR3 and the moderately thermophilic strain EPR7 were compared by determining the effect of temperature on the rates of Hg(II) volatilization by Hg(II)-induced resting cells (Fig. 3A). *E. coli* JM109/pKSM::Tn*501*

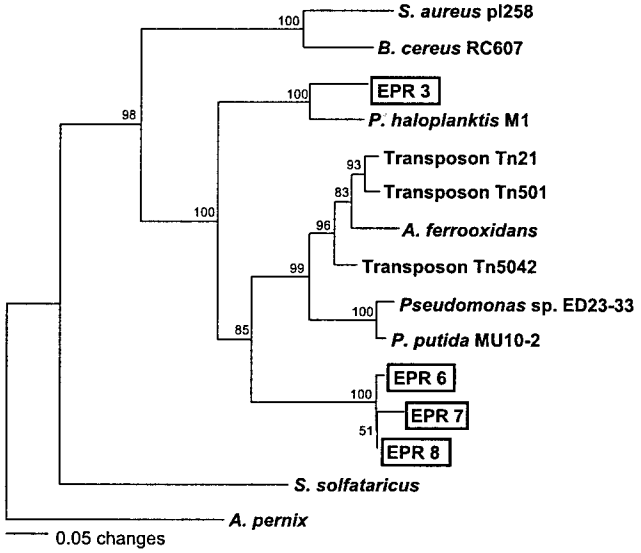

FIG. 2. Neighbor-joining tree for MerA amino acid sequences from deep-sea strains (boxes) and reference strains. The numbers at branching points are bootstrap values based on 100 replicates. The outgroup in the tree is the sequence of *A. pernix*.

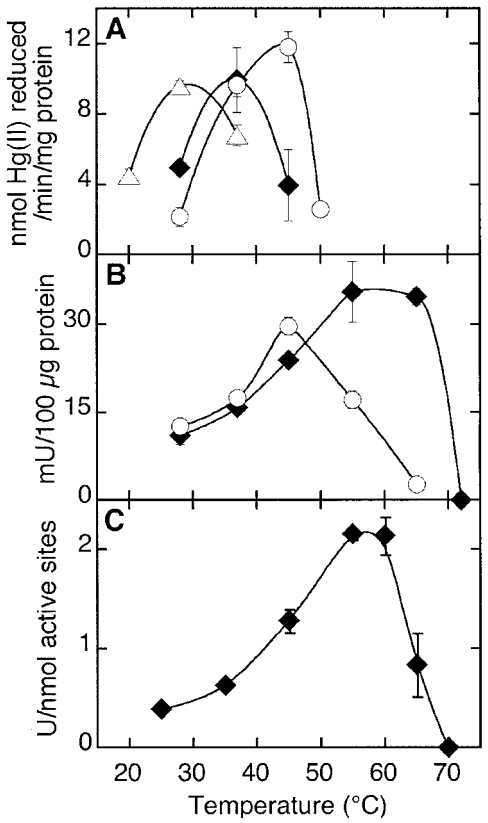

FIG. 3. (A) Effect of temperature on the specific rate of $^{203}HgCl_2$ volatilization by the activity of $HgCl_2$-induced resting cell suspensions. Means and standard deviations for four replicate samples are shown (for EPR3 observed rates were multiplied by a factor of 5) (△), JM109/pKSM::Tn*501* (◆), and EPR7 (○). (B) Effect of temperature on the specific rate of $HgCl_2$-dependent NADPH oxidation by crude cell extracts of $HgCl_2$-induced cultures. Means and standard deviations for two to four replicate samples are shown. Symbols: ○, EPR7; ◆, JM109/pKSM::Tn*501*. (C) Effect of temperature on the specific activity of purified Tn*501* catalytic core MR. Averages and ranges for two to four replicate analyses are shown.

served as a positive control in these experiments, since the MR that it encodes has been extensively characterized (13). The maximum rates of Hg(II) volatilization were observed at temperatures corresponding to the T_{opt} of the strains (Fig. 3A): 28°C for EPR3, 37°C for JM109/pKSM::Tn*501*, and 45°C for EPR7. The activities were completely inhibited at 45°C for EPR3 and at 55°C for both EPR7 and JM109/pKSM::Tn*501*. No Hg(II) volatilization was observed with uninduced cultures or uninoculated controls.

Because the MR is located in the cell cytoplasm (4), the temperature profiles of whole-cell Hg(II) volatilization activities may reflect the effect of temperature on the general metabolism of intact cells. The activities of MR in crude cell extracts were therefore determined at various temperatures for the moderately thermophilic vent strain EPR7 and for JM109/pKSM::Tn*501* (Fig. 3B). The temperature for optimal specific activity of the EPR7 enzyme, 45°C, corresponded to the optimal temperature measured in intact cells (Fig. 3A) and to the T_{opt} of this strain (Table 1). Unexpectedly, crude extracts containing the Tn*501*-encoded enzyme exhibited maximum activity between 55 and 65°C (35.6 ± 5.2 and 34.7 ± 1.5 mU/100 μg of extract protein, respectively), which was nearly 20 °C above both the temperature optimum for activity of intact cells (Fig. 3A) and the T_{opt} of the native strain in which Tn*501* was discovered (i.e., 37°C, at which the specific activity of crude cell extracts was 15.8 ± 0.7 mU/100 μg of extract protein).

To rule out the possibility that the temperature optimum for activities in crude extracts was due to the presence of temperature-sensitive inhibitors of MR (e.g., proteinases) rather than to the properties of the Tn*501* enzyme, the temperature profile of the purified catalytic core of Tn*501* MR was determined. The purified core enzyme, which has the same specific activity as the full-length enzyme (21), was optimally active at 55 to 60°C (2.2 ± 0.1 and 2.1 ± 0.2 U/nmol of active sites, respectively), became unstable at 65°C, and was totally inactivated at 70°C (Fig. 3C). At 35°C the core MR retained only 29% of its optimal activity (0.6 ± 0.0 U/nmol of active sites).

DISCUSSION

Data presented here show that mesophilic and moderately thermophilic bacteria obtained from various niches in a hydrothermal vent ecosystem were highly resistant to Hg(II) and that the resistance was most likely conferred by the enzyme MR. Furthermore, the discovery of a new cluster of MerA unique to moderately thermophilic, gram-negative vent strains expands the known diversity of this locus. Because selection for resistance to Hg(II) was not employed during primary isolation, the finding that >70% of the strains were resistant (Table 1) suggests that the microbial communities inhabiting the diffuse flow vent and associated plume were adapted to life in the presence of toxic concentrations of Hg. The distribution of mercury resistance among the deep-sea strains studied clearly showed that mesophilic and moderately thermophilic strains from the vent and its associated plume were more resistant to Hg(II) than psychrophilic strains collected at the control site. Thus, strains that were located closer to the source of the vent fluid were more resistant to Hg(II), supporting the hypothesis that the patterns of mercury resistance in the vent environment are niche dependent and possibly driven by mercury speciation. At this time, only one preliminary report showing highly elevated concentrations of Hg in hydrothermal vent fluid is available (8), but enrichment of Hg in sediments (10) and manganese nodules (7) collected in the vicinity of mid-ocean ridges has been known for a long time. In human-impacted ecosystems, enrichment of Hg(II)-resistant and -reducing bacteria that carry the *merA* gene is a hallmark of microbial communities exposed to mercury. In these environments, the activities of *mer*-carrying bacteria enhance removal of Hg(II) (5) and organomercury (30) as vapor Hg(0) to the atmosphere. Elemental Hg(0) is less toxic than Hg(II) (9), and the enrichment and activities of Hg(II)-reducing microorganisms may therefore play an ecological role in mercury detoxification, thereby contributing to the development of the rich fauna in deep-sea vents.

The elevated temperature optimum for the activity of the MR enzyme from Tn*501* (range, 55 to 65°C) (Fig. 3B and C) is characteristic of a thermophilic enzyme. The stability of MR from gram-negative bacteria at temperatures exceeding 80°C was reported previously; the Tn*501* enzyme retained full activ-

ity when it was tested at 37°C following a 10-min incubation at 100°C (24). However, MR activity at elevated temperatures was not reported previously. The thermophilicity of MR may be viewed as a relic of evolution in high-temperature environments based on the hypothesis that the first organisms were hyperthermophilic organisms (27) and the hypothesis that all extant life forms may have later adjusted to lower temperatures (1). It is intriguing that the optimal temperature of the MR that was isolated from a vent moderate thermophile, strain EPR7, was lower than that of Tn*501*'s MR (Fig. 3B). Interpretation of the phylogenetic analysis of MR (Fig. 2) in light of these data may lead to the hypothesis that the common ancestor of the clusters containing the Tn*501* and EPR7 MR was a thermophilic enzyme. In this scenario, it appears that the MR vent cluster evolved along a separate line of descent to the point where the optimal temperature for the catalytic activity of EPR7's enzyme coincides with the T_{opt} of the organism, 45°C. The finding that the optimal temperature for activity for Tn*501*'s MR is in the range from 55 to 65°C suggests that in the original mesophilic host of Tn*501*, catalysis by MR occurs at sufficient but suboptimal rates. More experiments, some currently in progress, are needed to test these hypotheses.

Deep-sea vents are likely ecological niches that are conducive to the evolution of metal resistance. However, as metal speciation is altered when hydrothermal fluids mix with oxygenated, cold seawater (20), vent biota are exposed to a gradient of metal toxicities. It was recently shown that sulfides alleviated metal toxicity in hyperthermophilic vent archaea by the formation of metal sulfides (12). Thus, selection for metal resistance in the vent ecosystem might be localized in niches exposed to cooler, more diluted vent fluids or in niches less affected by the large-scale precipitation of metal sulfides and oxides that occurs close to chimneys and vents (23).

It has been proposed previously that microbe-metal interactions evolved in geothermal environments, possibly in deep-sea hydrothermal vents (29). Evidence presented here supports this hypothesis by showing that there is enrichment of Hg-resistant bacteria in niches associated with deep-sea vents and by indicating that MR, the enzyme at the core of the broadly distributed mercury resistance system (4), is a thermophilic enzyme. Further examination of microbe-metal interactions in geothermal environments is needed to understand how toxicity in these naturally metal-enriched ecological niches affects evolution of metal resistance in the autochthonous microbial communities.

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